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Original Article

A Cell Line Assay System for Predicting the Response of Human Blood to Endotoxin

Akihiko Yamamoto*, Takeo Sakai[1], Masaki Ochiai, Kazunari Kamachi, Michiyo Kataoka, Hiromi Toyoizumi and Yoshinobu Horiuchi

Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Tokyo 208-0011 and
[1]Department of Veterinary Medicine,
College of Bioresource Sciences, Nihon University, Kanagawa 252-8510, Japan

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SUMMARY: Some parenteral drugs augment the in vivo action of endotoxin. It is necessary to regulate the overall toxic action of contaminating endotoxin by developing a clinically relevant test method for the safety control of such drugs. Although the responses of human peripheral blood cells (hPBC) to endotoxins to produce tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and IL-1β showed considerable variation depending on the endotoxin and also on the individuals used as sources of blood, the responses to each of the endotoxins evaluated relative to that to the Japanese reference standard endotoxin were found to be highly reproducible irrespective of the sources of hPBC. The evaluation procedure based on the relative responsiveness to various endotoxins was shown to be highly effective to detect differences in responsiveness among the endotoxin test, the pyrogen test, and the cytokine induction in hPBC. When eight human monocytoid cell lines were examined, only THP-1 and 28SC cells showed a significant dose-dependent IL-6 production. However, THP-1 failed to show consistency with hPBC in responses to the panel of endotoxins. 28SC cells showed appropriate consistency with hPBC not only in terms of respective responses to the endotoxins but also with regard to detection of the effect of human interferons to augment endotoxin to induce IL-6.

INTRODUCTION

Endotoxin is known to have various harmful biological activities (1-3). It causes febrile reactions in humans and rabbits by a small amount (4). Contamination by endotoxin, therefore, is a serious threat for the safety of parenteral drugs. The pyrogen test in rabbits played a crucial role in control of endotoxin in pharmaceuticals (5-7). In recent years, this test has been replaced by the endotoxin test, for a wide range of pharmaceutical products (5-9). The endotoxin test is highly sensitive and more accurate than the pyrogen test (9). However, the endotoxin test cannot predict the possibility of parenteral drugs augmenting in vivo actions of endotoxin (10-13). Therefore, a test method to directly evaluate the in vivo actions of endotoxin, including in vivo synergism, is necessary to establish effective safety control of such drugs. We previously reported a highly sensitive in vitro test method for endotoxin activity, using a mouse monocytoid cell line, RAW264.7 cells, that could detect a synergistic effect of human interferon alpha (hIFN-α) on endotoxin (10). However, due to possible species-specificity of drugs, that test method is still assumed to have limited efficacy for evaluating the synergistic effect (14). A test method using a cell line of human origin would be adequate for evaluating the overall toxic action of contaminated endotoxin in such drugs.

Ex vivo or in vitro substitutes for the pyrogen test using human whole blood or cell lines of human origin have been reported by some workers (15-18). Although the test methods were reported to have sufficient consistency with the pyrogen test in rabbits and also with the endotoxin test, the pyrogens used for the evaluations were only combinations of extreme examples; they were not adequate for sensitive detection of differences between test methods (15-18).

Endotoxins from different sources were reported to have different strength of Limulus amoebocyte lysate (LAL) activating activity (LAL activity) and pyrogenicity in rabbits relative to body weight (24). Further, the relationship between LAL activity and pyrogenicity in rabbits of endotoxin was shown to vary depending on the source of endotoxin (24). We employed the relative activities of a panel of endotoxins from various sources, referring to the activities of the Japanese reference standard endotoxin for the sensitive detection of difference of test system in the present study. We first ensured the effectiveness of using the relative activities of a panel of endotoxins from various sources for comparing test methods and subsequently identifying an adequate test method for predicting clinical satety.

We herein present the results of a precise examination of eight different human monocytoid cell lines, conducted using a quantitative method of evaluation to select a cell line that has a responsiveness similar to that of human peripheral blood cells (hPBCs) including the capability to detect a synergistic effect between drugs and endotoxin.

MATERIALS AND METHODS

Endotoxins: Japanese National Reference Standard Endotoxin Lot 3 (RSE) extracted from *Escherichia coli* UKT-B strain (13,000 endotoxin units [EU] per vial) (19) and commercial endotoxins extracted from *E. coli* O111: B4, *E.*

*Corresponding author: Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan. Tel: +81-42-561-0771, Fax: +81-42-567-0740, E-mail: yama-aki@nih.go.jp

coli O55: B5, *E. coli* O127: B8, *Salmonella typhosa* O901, *S. enteritidis* (Difco Laboratories, Detroit, Mich., USA), *S. abortus equi*, *Pseudomonas aeruginosa* serotype 10, *Klebsiella pneumoniae*, and *Vivrio cholerae* serotype Inaba 569B strain (Sigma Chemical Co., St. Louis, Mo., USA) by the Westphal's phenol water method were used. RSE was reconstituted in 1.3 ml of pyrogen-free distilled water for injection (5) (Otsuka Pharmaceutical Co., Ltd., Tokyo) before use. The commercial endotoxins were suspended in pyrogen-free distilled water for injection at a concentration of 1.0 mg/ml, and kept at 4°C, without detectable change in LAL activity for more than a year, until use.

Biological products: Five batches each of commercially available hIFN-α, -β, and -γ injections (Sumitomo Pharmaceuticals Co., Ltd., Osaka, Otsuka Pharmaceutical, Mochida Pharmaceutical Co., Ltd., Tokyo, Toray Industries, Inc., Tokyo, or Suntory Pharmaceuticals Inc., Osaka) were purchased from the market. Specific activity of the batches varied from 1,000,000 to 6,000,000 units per vial, and the contents were used by diluting serially with physiological saline (Otsuka Pharmaceutical). Vial contents were proved to be free of detectable endotoxin contamination at the concentration of 600,000 units per milliliter, as examined by the endotoxin test, according to the Minimum Requirements of Biological Products of Japan (MR) (20).

Pyrogen test: Female rabbits of the Japanese white strain (Kitayama Labes Co., Ltd., Nagano, or Japan Laboratory Animals, Inc., Tokyo) weighing approximately 3 kg were housed in individual cages at around 23°C in an air-conditioned animal room. Three animals were allocated for each treatment, unless otherwise stated. They were intravenously injected with 1 ml/kg of a dilution of test samples. Rectal temperatures of the animals were monitored for the next 3 h using an electric thermometer (Scanner Unit X115 with High Accurate Data Logger K730, TECHNOL SEVEN, Kanagawa). Pyrogenicity of a test sample was calculated relative to that of the RSE according to the parallel line assay method using logarithmic doses and the maximum rise in rectal temperature during a 3-h period.

Cell lines: Human monocytoid cell lines THP-1 (ATCC TIB 202), P31/FUJ (JCRB0091), P39/TSU (JCRV0092), MD (ATCC CRL 9850), 90196B (ATCC CRL 9853), EL1 (ATCC CRL 9854), 28SC (ATCC CRL 9855), and KMA (ATCC CRL 9856) were used. THP1, P31/FUJ, and P39/TSU cells were maintained in RPMI 1640 medium (Dainippon Pharmaceutical Co., Ltd., Tokyo) supplemented with 2 mM of L-glutamine (Dainippon Pharmaceutical) and 10 (v/v)% foetal calf serum (HyClone Laboratories Inc., Logan, Utah, USA) at 37°C in a humidified atmosphere of 5% CO_2 / 95% air. Iscove-modified Dulbecco's modified Eagle's medium (SPI, Tokyo) supplemented with 10 (v/v)% foetal calf serum was used for maintaining the other cell lines. 28SC was initially maintained with Iscove-modified Dulbecco's modified Eagle's medium, and the medium was later replaced with RPMI1640 that was more nutritious and was expected to improve growth conditions for 28SC cells. 28SC cells showed increased sensitivity to endotoxin from approximately 10 EU/ml to 0.1 EU/ml during 40 subcultures, which finding is similar to that in a previous report on RAW264.7 cells (10).

In vitro hPBC assay: Peripheral blood samples were collected from healthy donors after obtaining their informed consent. A 100 μl-volume of heparinized fresh human peripheral blood was mixed with 900 μl of a dilution of test samples or RSE and incubated at 37°C. Kinetics analysis revealed that interleukin 1 beta (IL-1β), IL-6, and tumour necrosis factor alpha (TNF-α) productions reached maximum levels at around 9 h of incubation, and showed no change thereafter up to 50 h. Accordingly, incubation for 18 h was selected for the routine assay. Supernatants of the mixtures were isolated by a centrifuge to determine IL-1β, IL-6, and TNF-α concentrations by enzyme-linked immunosorbent assay methods (ELISA) (BD PharMingen, San Diego, Calif., USA). The measurement was repeated twice using blood from two different donors, and the results were calculated relative to the activities of the RSE and expressed as geometric means of the relative activities obtained in the repeat measurements.

In vitro IL-1β, IL-6, and TNF-α induction assays in human monocytoid cell lines: Each of the cell lines was cultured and the concentration was adjusted to 1×10^6 cells/ml. A 100 μl of each type of cultured cells was transferred to wells of a 96-well tissue culture plate (Nunclon D, Nalge Nunc International Co., New York, N.Y., USA). The cells were stimulated with a 100-μl of a dilution of the test samples 72 h later. In an experiment to investigate in vitro synergism, the cells were stimulated with a 100 μl-volume of appropriate dilutions of a mixture of RSE and a hIFN injection. Culture supernatants were isolated 18 h after the stimulation to assay IL-1β, IL-6, and TNF-α concentrations by ELISA systems (BD PharMingen) so as to allow a comparison with hPBC under the same condition.

The bacterial endotoxin test: The suspensions of endotoxins were serially diluted with pyrogen-free distilled water at fourfold intervals. A 50-μl volume each of the appropriate dilutions was mixed with an equal volume of an endotoxin-specific LAL reagent (Endospecy, Seikagaku Corp., Tokyo) whose reactivity to (1-3)-β-D-glucan had been removed (21). Rate of color development was measured using a specially equipped micro-plate reader (Well Reader SK601, Seikagaku Corp.) (22). Endotoxin contents of samples were calculated according to the parallel line assay method using the logarithmically transformed dose and the rate of color development, and expressed as EU/ml referring to RSE.

Statistical analysis: Relative activity was estimated according to the parallel line assay method (23). Calculation of confidence limits and significance testing were made at a level of $P = 0.05$.

RESULTS

Pyrogenicity in rabbits and cytokine-induction activity in hPBC of endotoxins from various sources: We employed a panel of endotoxins from various sources to compare test methods. The endotoxins were serially diluted with physiological saline at fivefold intervals from 300 to 0.48 EU/ml, according to their endotoxin test results. hPBC culture was stimulated with the dilutions in order to measure IL-1β, IL-6, and TNF-α releases in culture fluid, and to evaluate the cytokine-induction activities of each endotoxin relative to those of RSE by the parallel line assay method. The assay was repeated using hPBC from two different donors. Although hPBC from different donors showed considerable variation in production of the cytokines in response to endotoxins, cytokine inductions by each of the endotoxins relative to those by RSE were found to be highly reproducible in repeat measurements. Accordingly, the activities of endotoxins to induce IL-1β, TNF-α, and IL-6 were calculated as geometric means of the relative activities obtained in repeat measurements. Relative activities of IL-1β, IL-6, and TNF-α induc-

tion varied considerably depending on the source of endotoxin in spite of the fact that the comparison was conducted at a homogeneous level of LAL activity, suggesting a discrepancy between LAL activity and cytokine induction in hPBC. Geometric mean values of repeat measurements of IL-6 induction activities of the endotoxins, relative to that of RSE, are shown in Fig. 1 as an example.

Among the endotoxins tested, that from *S. entritidis* showed the strongest activity of IL-6 induction. Endotoxin from *K. pneumoniae* was found to be more potent in TNF-α induction than endotoxin of *S. entritidis* (data not shown). Endotoxins from *S. typhosa* 0901, *P. aeruginosa*, and *V. cholerae* Inaba 569B induced only trace levels of the cytokines. Induction profiles of IL-1β and TNF-α in hPBC in response to endotoxins from various sources were found to be quite similar to that of IL-6. Fine correlations were seen between TNF-α and IL-6 (r = 0.945 [$P < 0.0001$]) and between IL-1β and IL-6 (r = 0.914 [$P = 0.0002$]) inductions (Fig. 2). The relationship of induction profiles between the cytokines was used, hereafter, for comparisons with other assays to establish the characteristics of hPBC.

The endotoxins were serially diluted to 270, 90, and 30 EU/ml, and their pyrogenicity was assayed in rabbits to calculate the pyrogenicity of each endotoxin relative to that of RSE according to the parallel line assay method. Quantitatively measured pyrogenicity of the endotoxins showed considerable variation in spite of the fact that the comparison was conducted at homogenous levels of LAL activity (data not shown). The pyrogenic response of rabbits and the cytokine production of hPBC in response to the panel of endotoxins were compared by correlation analysis. Although a good correlation has been previously reported (15,16), correlation coefficients between the pyrogenic response of rabbits and IL-1β, IL-6, and TNF-α productions of hPBC were 0.0082 ($P = 0.982$), 0.147 ($P = 0.684$), and 0.279 ($P = 0.433$), respectively; showing no clear correlation. Accordingly, the comparison using endotoxins from various sources was considered to be a sensitive method for detecting differences among test methods, and, therefore, an adequate measure for evaluating the relevance of test methods to responsiveness of hPBC.

A cell line test system for endotoxin that has a responsiveness similar to that of hPBC: Previous researchers have reported on indicator cell line assays that were evaluated using combinations of pyrogens (15-19,25). However, the combinations of pyrogens were adequate only for detecting extreme differences, not for making a detailed evaluation or a sensitive detection of differences between test systems. We searched for a cell line that exhibited a responsiveness similar to that of hPBC by making detailed examinations, using a panel of endotoxins.

We first examined IL-6 production in eight different monocytoid cell lines of human origin in response to stimulation with graded concentrations of RSE, measuring IL-6 in the culture fluid after incubating for 18 h as shown in Fig. 3. Cell lines 90196B, KMA, P31/FUJ, and P39/TSU showed no significant production of IL-6 over the control levels in response to stimulation with RSE at concentrations up to 5,000 EU/ml. The cell line MD was a spontaneous producer of a rather high level of IL-6 irrespective of stimulation. THP-1 cells showed a clear dose-dependent response to RSE at concentrations between 2.0 EU/ml and 250 EU/ml, producing IL-6 at levels approximately ranging from 5 to 130 pg/ml. Among the cells examined, 28SC cells responded most sensitively to produce IL-6, at levels ranging from 9.6 to 520 pg/ml in response to graded concentrations of RSE from 0.4 EU/ml to 50 EU/ml. EL1 showed IL-6 production at a level significantly higher than that of the control, but at a level much lower than that of THP-1 or 28SC cells in response to RSE of over 40 EU/ml.

We carried out further examinations of TNF-α and IL-1β

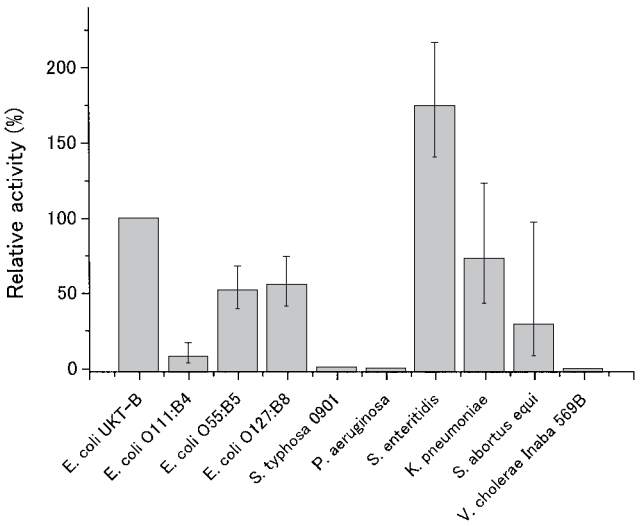

Fig. 1. Cytokine-induction activity in hPBCs of endotoxins from various different sources relative to that of RSE.
The endotoxins were serially diluted and added to hPBC culture to measure the production of IL-6 using an ELISA method. IL-6 induction activity of each endotoxin was calculated relative to that of RSE according to the parallel line assay method. Although the levels of IL-6 production showed considerable variation in repeat measurements using blood from two different donors, the relative activity of each of the endotoxins was found to be quite reproducible. Accordingly, IL-6 induction activity of each endotoxin was shown as the geometric mean and standard deviation of the repeat measurements of relative activity. The relative IL-6 induction activity of endotoxins showed considerable variation in spite of the fact that the comparison was based on homogenous levels of LAL activity.

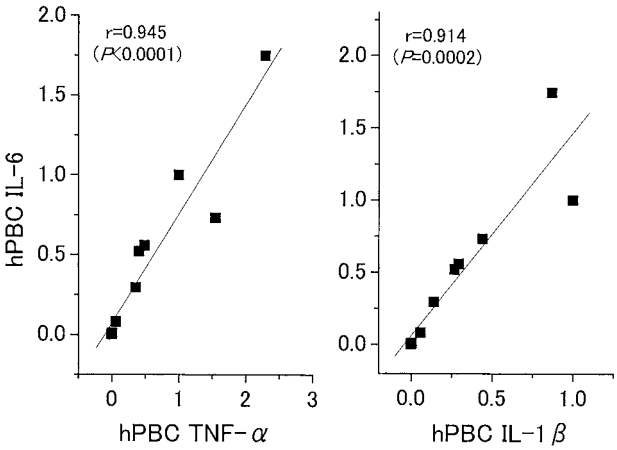

Fig. 2. Relationship between induction profiles of IL-6, IL-1β, and TNF-α by endotoxins from various sources in hPBCs.
Activities of the endotoxins to induce IL-6, IL-1β, and TNF-α in hPBCs were measured relative to the activities of RSE, in the similar way to that for Fig. 1. One particular characteristic of the responsiveness of hPBCs was shown to be a strong similarity of the induction profiles of IL-1β and TNF-α to that of IL-6 in response to endo-toxins from various sources.

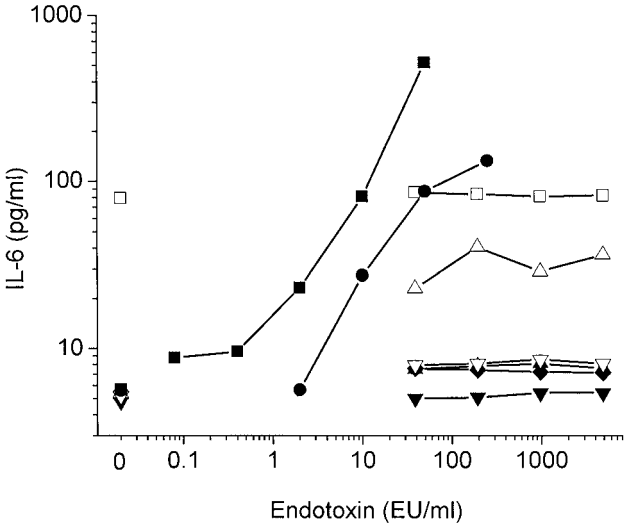

Fig. 3. IL-6 production of human monocytoid cell lines in response to graded concentrations of endotoxin.
Cultures of eight different human monocytoid cell lines were stimulated with graded concentrations of RSE to measure the production of IL-6 in culture supernatants 18 h later according to the parallel line assay method. Cells without RSE stimulation, used as the controls, were examined in parallel. Cell lines 90196B (▲), KMA (▽), P31/FUJ (▼), and P39/TSU (◆) showed no significant dose-dependent response to RSE to produce levels of IL-6 over the control levels. The cell line MD (□) spontaneously produced a significant level of IL-6 without stimulation, and showed no dose-dependent change in IL-6 level. Although THP-1 (●) and 28SC (■) cells showed clear dose-dependent responses to RSE, 28SC cells were found to be the most sensitive cell line among those tested. EL1 cells (△) produced IL-6 at a much lower level than THP-1 or 28SC but at a lelvel significantly higher than that of the control.

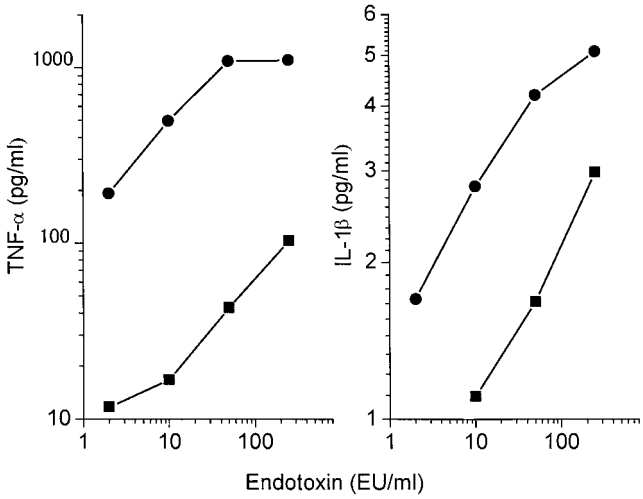

Fig. 4. IL-1β and TNF-α productions by THP1 cells and by 28SC cells in response to endotoxin.
THP-1 and 28SC cells were stimulated with graded concentrations of RSE to measure the concentrations of IL-1β and TNF-α in the culture supernatants 18 h later. THP-1 (●) cells showed a highly sensitive response to endotoxin, producing up to 1,100 pg/ml TNF-α in response to 50 EU/ml of RSE. 28SC (■) cells also showed TNF-α production in a dose-dependent manner. Both THP-1 and 28SC cells showed dose-dependent IL-1β production, but at a level much lower than that of TNF-α.

productions in THP-1 and 28SC cells according to the results shown in Fig. 3. THP-1 cells produced approximately 190 pg/ml of TNF-α in response to 2.0 EU/ml of RSE, increasing production up to 1,100 pg/ml depending on increase in the stimulus up to 50 EU/ml (Fig. 4). 28SC cells produced 12 pg/ml of TNF-α in response to 2.0 EU/ml of RSE, increasing production up to 100 pg/ml with increasing in endotoxin to 250 EU/ml. In terms of IL-1β, both THP-1 and 28SC cells showed a dose-dependent response similar to that of TNF-α production, in which THP-1 responded more sensitively than 28SC cells.

THP-1 and 28SC cells were further evaluated in terms of their responses to endotoxins from various sources to produce IL-6, TNF-α, and IL-1β, which were compared to the responses of hPBC. Correlation coefficients between THP-1 cells and hPBC to produce IL-6, TNF-α, and IL-1β in response to the panel of endotoxins were 0.593 (P = 0.071), 0.097 (P = 0.789), and −0.159 (P = 0.660), respectively (Fig. 5a). In spite of previous report of similarity to hPBC (26), the responsiveness of THP1 cells was proved to be quite different from that of hPBC, whereas 28SC cells showed clear correlations with hPBC in terms of productions of IL-6, TNF-α, and IL-1β, as shown in Fig. 5b. Correlation coefficients between 28SC cells and hPBC to produce IL-6, TNF-α, and IL-1β in response to the panel of endotoxins were 0.930 (P < 0.0001), 0.718 (P = 0.0194), and 0.682 (P = 0.0299), respectively. The correlations of TNF-α and IL-1β productions could be improved by eliminating the results of *E. coli* O55:B5 for TNF-α and RSE for IL-1β significantly, to 0.950 (P < 0.0001) and 0.982 (P < 0.0001), respectively.

Responses of THP-1 and 28SC cells to the panel of endotoxins to produce the cytokines were further compared with those of hPBC. When the activities of endotoxins to induce IL-6, TNF-α, and IL-1β were measured relative to the activities of RSE, hPBC showed clear correlations between production profiles of the cytokines, as shown in Fig. 2, whereas THP-1 cells showed no such correlation between IL-6 and TNF-α (r = −0.0195 [P = 0.957]) or between IL-6 and IL-1β (r = 0.558 [P = 0.094]) productions in response to the endotoxins. On the other hand, 28SC cells showed clear correlations, similarl to those of hPBC, between IL-6 and TNF-α (r = 0.907 [P=0.0003]) and also between IL-6 and IL-1β (r = 0.931 [P < 0.0003]) productions. Relationships of IL-6 and TNF-α productions in THP1 and 28SC cells are represented in Fig. 6 as examples.

Although the previous report found a good correlation between THP1 and hPBC (26), detailed analysis in the present study revealed that THP1 and hPBC have quite different responsiveness to endotoxins. On the other hand, the responsiveness of 28SC cells was shown to be consistent with that of hPBC. According to the detailed evaluation, we chose IL-6 production in 28SC cells as the most sensitive and appropriate cell line assay system for detecting endotoxin activity.

Detection of synergistic effect between hIFNs and endotoxin: Effect of hIFN injections on endotoxin activity was examined in hPBC and 28SC cells. Serial concentrations of hIFN-α, hIFN-β, and hIFN-γ were respectively mixed with graded concentrations of RSE at fivefold intervals and added to cultures of hPBC and 28SC cells in order to examine IL-6 induction. The same dilutions of RSE without addition of hIFNs, and 10^4 U/ml of the hIFNs without RSE were examined in parallel as the controls. IL-6 in the culture supernatants was determined by an ELISA after incubation at 37°C for 18 h. Doses of RSE used for stimulating hPBC were 12.5, 2.5, 0.5, and 0.1 EU/ml and those used for 28SC cell were 50, 10, 2, and 0.4 EU/ml. IL-6 induced by the graded doses of the control RSE in hPBC were 702.6, 431.2,

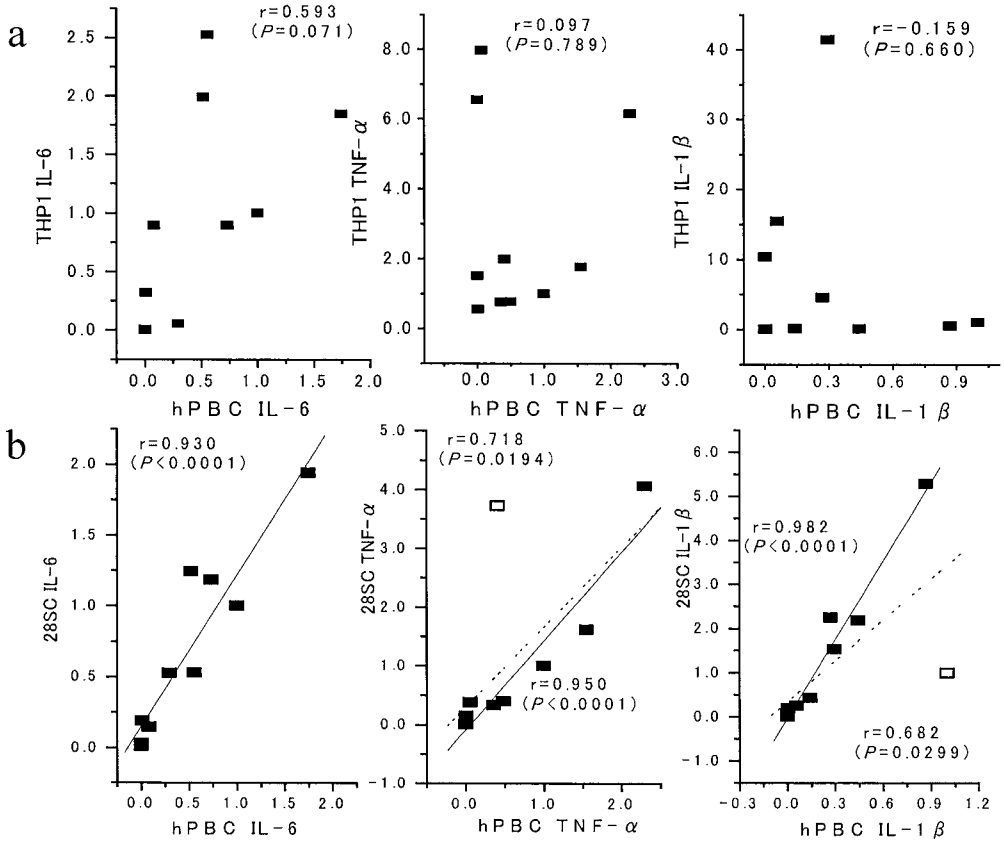

Fig. 5. Proinflammatory cytokine inductions by endotoxins from various sources in THP-1 and 28SC cells compared with those in hPBCs. The activities of endotoxins from various sources to induce IL-6, TNF-α, and IL-1β in THP-1 and 28SC cells were measured relative to the activities of RSE. Profiles of cytokine productions in THP-1 and 28SC cells in response to the endotoxins were compared with those in hPBCs. Responses of THP-1 and hPBCs were dissimilar. 28SC cells, however, showed clear correlations to hPBCs in terms of productions of IL-6, TNF-α, and IL-1β, proving the similarity of their responsiveness. Correlations between hPBCs and 28SC cells in terms of TNF-α and IL-1β productions could be improved considerably by excluding the extreme results of *E. coli* O55:B5 (□) and RSE (□) from analysis, respectively.

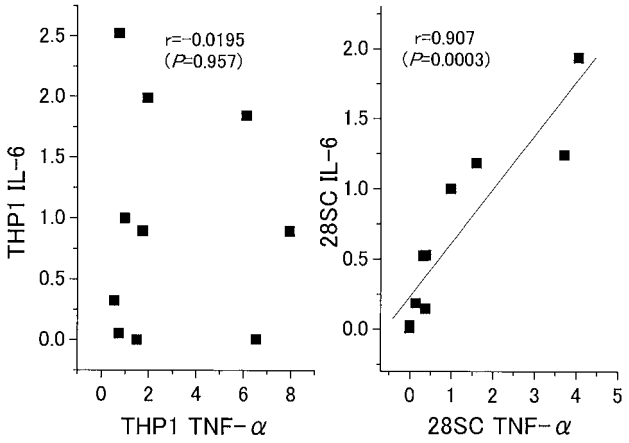

Fig. 6. Comparison of induction profiles of proinflammatory cytokines in THP1 and 28SC cells by endotoxins from various sources. Activities of the endotoxins to induce IL-6 and TNF-α were assayed in THP-1 and 28SC cells relatively to the activities of RSE. In contrast with hPBCs, no consistent relationship was seen between IL-6 and TNF-α productions by the endotoxins in THP-1 cells. On the other hand, 28SC cells showed a clear correlation between IL-6 and TNF-α productions in response to the endotoxins, suggesting a responsiveness similar to that of hPBCs, as shown in Fig. 2.

263.6, and 23.6 pg/ml, respectively, and those induced by 10^4 U/ml of the control hIFN-α, hIFN-β, and hIFN-γ without RSE were 8.3, 9.1, and 9.4 pg/ml, respectively. However, 12.2 pg/ml of IL-6 was detected in the supernatant of hPBC culture in the absence of RSE or hIFNs stimulation. Therefore, the IL-6 detected after stimulation with hIFNs alone could be considered negligible. The graded concentrations of RSE induced 521.7, 84.4, 23.2, and 9.5 pg/ml, respectively, of IL-6 in 28SC cells, whereas no excess IL-6 over the detectable limit of 4.6 pg/ml was induced by any of the hIFNs without endotoxin, even at 10^4 U/ml. IL-6 induced by the mixtures of dilutions of hIFNs and RSE was compared to that induced by the control RSE in order to estimate the relative effect of enhancement quantitatively. The hIFNs mixed with RSE enhanced IL-6 induction in both types of cells in a dose-dependent manner, as shown in Fig. 7. Further, the responses of hPBC and 28SC cells to hIFN-α, hIFN-β, and hIFN-γ were shown to be quite similar, suggesting again that the 28SC cell line is an appropriate indicator of the responsiveness of human blood cells.

It is crucial for the safety control of interferon injections to regulate not only the amount of contaminated endotoxin but also the adverse reactivity to it in humans. Based on the finding that 28SC cells were found to have responsiveness consistent with that of hPBC, we attempted a quantitative evaluation of the enhancement effect of hIFNs on endotoxin activity to induce IL-6, using 28SC cells. 10^4 U/ml of hIFN-α, hIFN-β, or hIFN-γ was added to graded concentrations of RSE from 50 EU/ml to 0.4 EU/ml, and IL-6 induction in 28SC cells was assayed. Results were compared with those

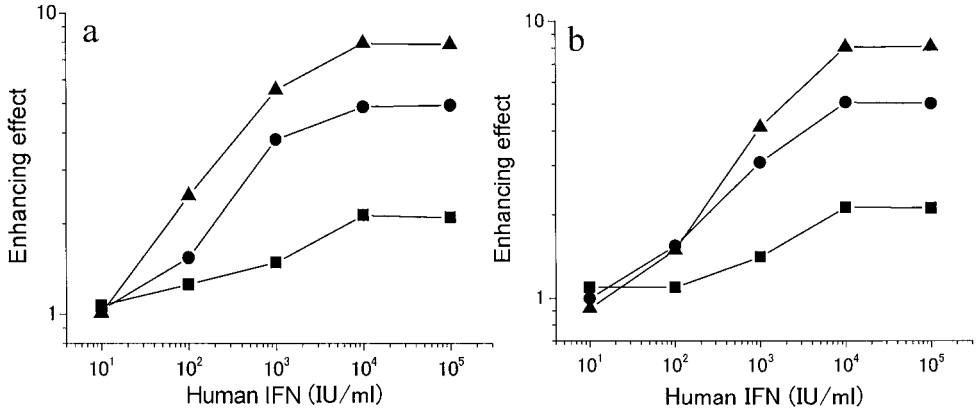

Fig. 7. Dose-dependent effect of hIFN-α, hIFN-β, and hIFN-γ to augment endotoxin to induce IL-6 in hPBCs and in 28SC cells. hPBCs and 28SC cells were stimulated with serial dilutions of RSE ranging from 12.5 to 0.1 EU/ml or from 50 to 0.4 EU/ml, respectively. The cells were also stimulated with a mixture of a dilution of RSE and varied concentrations of IFNs, as indicated in the figure, or with 10^4 U/ml of hIFNs without RSE, to measure IL-6 production. Enhancement effects of IFNs were calculated relative to the response to RSE alone. IFN-α (●), IFN-β (■), and IFN-γ (▲) augmented IL-6 induction of RSE both in hPBC (a) and in 28SC cells (b) in a highly consistent manner.

Table 1. Effect of human IFNs to augment endotoxin to induce IL-6

Sample	Cell	Enhancement effect[1)]	±2SD[2)]
Control endotoxin		1.000	
Endotoxin + IFN-α	28SC	2.173	(1.980 - 2.367)
	hPBC	2.126	
Endotoxin + IFN-β	28SC	5.227	(4.874 - 5.580)
	hPBC	5.097	
Endotoxin + IFN-γ	28SC	7.938	(7.509 - 8.367)
	hPBC	7.942	

[1)]: Caliculated relatively to the control endotoxin.
[2)]: measured on 5 batches.

for induction of IL-6 by the same concentrations of the control RSE without hIFNs. The dose-responses of all the samples were parallel, and the enhancement effect of hIFNs could be evaluated as potencies of IL-6 induction relative to those of the control RSE according to the parallel line assay method (data not shown).

We applied this method for evaluating the enhancement effect of five batches each of commercial hIFN-α, hIFN-β, and hIFN-γ injections on IL-6 induction by RSE. For validation, the results were compared with that in hPBC. Results are summarized in Table 1.

The batches of hIFN-α were shown to enhance the activity of RSE to induce IL-6 by approximately 2.2-fold with only slight batch-to-batch variation in enhancement activity. The extent of enhancement was almost similar to that in hPBC, as shown in the table. Batches of hIFN-β and hIFN-γ injections showed enhancement effects of approximately 5.1- and 7.9-fold, respectively, and also showed only slight batch-to-batch variation. The enhancement effects in 28SC cells were again shown to be consistent with those in hPBC.

DISCUSSION

Some parenteral drugs augment in vivo biological activities of endotoxin (27). Effective safety control tests for endotoxin contamination in such drugs require not only quantifying endotoxin but also regulating the overall in vivo action of contaminating endotoxin. In particular, drugs that showed species specificity for synergistic enhancement would require special caution when interpreting the results of control tests (14). Neither the current pyrogen test nor the endotoxin test is adequate for predicting the clinical safety of such drugs if the synergistic effect is not taken into appropriate consideration (10). It is, therefore, assumed crucial to establish an assay method that can effectively predict the overall response in humans, including the effect of augmentation of contaminated endotoxin in pharmaceuticals.

Fennrich et al. (28) and Pool et al. (29) reported assay methods using peripheral human blood. However, an assay using human blood might present safety or ethical difficulties. As alternatives, human monocytoid cell lines were examined as potential indicator cells for testing pyrogenic substances in medical products (17,18,25,26). Although the cell line assays were reported to be effective to detect pyrogens, the validation procedures employed in those reports were not sufficient to prove consistency of responsiveness of the cell lines with that of humans.

We compared the results of the pyrogen test, the endotoxin test, and the assay of proinflammatory cytokine inductions in hPBC using endotoxins from various sources, and found that the results of those tests were not consistent with each other. Activities to induce IL-6, TNF-α, and IL-1β in hPBC varied considerably depending on the source of endotoxin, even though the measurements were performed at homogeneous levels of LAL activity. Pyrogenicity in rabbits was shown to be inconsistent not only with LAL activity but also with the cytokine inductions in hPBC, based on evaluation using the panel of endotoxins from various sources. These findings suggest that there are considerable differences in responsiveness among LAL, rabbits, and hPBC, and, therefore, that the endotoxin test and the pyrogen test have limited efficacy for controlling the safety of medical products. In addition, the discrepancies between the results of the present study and those of previous studies mentioned above suggest that the procedure using the panel of endotoxins employed in the present study was very sensitive for discriminating the characteristics of different assay methods, and, accordingly, is expected to be useful for identifying a test method with the responsiveness similar to that of hPBC.

We examined eight different human monocytoid cell lines using a panel of endotoxins from various sources, and found that 28SC cells have a responsiveness to endotoxins quite

similar to that of hPBC. Further, 28SC cells were shown to be effective for predicting the effect of interferons to augment the induction of IL-6 by endotoxin in hPBC. Accordingly, determination of IL-6 production in 28SC cells was selected as the most appropriate indicator cell line assay for controlling endotoxin in parenteral drugs.

Discrepancies among responses to endotoxins of LAL, rabbit, and hPBC were considered to reflect differences in the responsiveness of the species for the sources of the assay systems. Further, hPBC showed IL-6, TNF-α, and IL-1β productions that clearly correlated with each other, and we used these production profiles to identify the relevant cell line assay system. One interesting question raised was whether such consistency among cytokine inductions in response to the panel of endotoxins is a common characteristic for any animal species. Moreover, among the cell lines examined, only 28SC but not THP1 was found to maintain characteristics similar to those of hPBC for cytokine productions in response to the panel of endotoxins. Although the explanation for the similarity between 28SC and hPBC, and the difference between 28SC and THP1 are unknown, the latter is interesting in that it reflects a difference in endo-toxin receptors or in transduction mechanisms.

Some discrepancies regarding relationships among the endotoxin test, pyrogen test, and cytokine induction assays in hPBC exist between the findings in the present study and those in previous studies (16,17,25). Further, the cytokine induction assay in THP-1 cells that was reported to correlate with the endotoxin test and the pyrogen test showed no correlations in the present study. Other than differences between the pyrogens we used for the evaluations and those used in previous studies, differences in accuracy of test results between the present study and previous reports might be another reason for the discrepancies. Previous researchers employed semi-quantitative results of the tests, which might not be adequate for a reliable correlation analysis. In the present study, in spite of previous reports of good correlations, we employed quantitative methods for all the tests in order to obtain the most accurate evaluations (15, 16).

Although the endotoxin test has the efficacy limitations described above, it could still be used effectively in routine control tests if validated appropriately to revise its current endotoxin limit values. The current regulations for endotoxin contamination, particularly in biological products, need to be revised to effectively control for adverse reactivity in humans. The IL-6 induction assay in 28SC cells could be used for an effective validation of the current endotoxin limits for biological products. Regulation should be set to regulate endotoxin content to a level below that at which no any practically available endotoxin would show excess IL-6 induction in 28SC cells over the acceptable level for RSE alone.

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